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Relationship of Microbial Activity to Changes in Lipids of Foods

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Contents

1. Introduction

Most foods contain significant amounts of fat, and these have long been recognized as susceptible to hydrolysis, oxidation and other chemical processes that result in the formation of new compounds. Both desirable and undesirable flavour changes in food products are associated with these compounds, although definite causal relationships are sometimes difficult to establish. In a similar manner, 'guilt by association' has assigned to many micro-organisms a role in lipolysis which may not be entirely correct. For example, the number of lipolytic bacteria found in a product often has been associated with rancidity development or change in flavour (Hammer & Collins, 1934; Lea, 1938; Lawrence, 1967*a*, *b*) even though the measurement of bacterial lipolysis was for hydrolysis while the off flavour or odour was caused by oxidation. In addition, the fat or triglyceride in the bacterial medium may have a quite different composition from the fat in the product of interest.

In this paper we have interpreted 'lipolysis' to mean any fat decomposition. This includes not only hydrolysis of the triglyceride to di- and mono–glycerides and fatty acids, but also oxidative changes of the intact triglycerides or of the component fatty acids after preliminary hydrolysis. Data from our own and other laboratories will be examined in an attempt to more clearly define the actual and potential role of micro-organisms in the total lipolytic changes in foods. For convenience, hydrolytic and oxidative changes will be discussed in separate sections.

2. Hydrolytic Changes

(a) *Definition*

The ability of many micro-organisms to hydrolyse triglycerides and natural fats has been a matter of concern for many years, and the generic name 'lipase' is usually given to the enzyme responsible for this hydrolysis. On the basis of arguments set forth by Desnuelle & Savary (1963) and Wills (1965), the International Union of Biochemistry (1965) has recommended that the designation 'true lipase' be reserved for those enzymes that attack only insoluble substrates. We have supported this contention (Alford, Pierce & Suggs, 1964; Mencher & Alford, 1967). Others have included enzymes attacking soluble substrates (Hobson & Summers, 1966; Lawrence, Fryer & Reiter, 1967) in their definition. Lawrence (1967*a*, *b*) effectively summarized the numerous studies of microbial lipases involving all kinds of measurements; he accepted the insoluble substrate as somewhat definitive simply because these are the most common natural substrates. Regardless of whether the substrate used for lipase detection is soluble or insoluble, the end products are usually the fatty acids (FFA) resulting from hydrolysis of ester linkages. The detection of these FFA is either by observing colour changes or clearing around colonies in a Petri plate so that the significant micro-organisms can be isolated and enumerated, or by chemical means for quantitation and identification of the FFA.

(b) *Detection of lipase—producing micro-organisms*

Many methods have been proposed for the visual detection of lipase production; most of them depend upon changes in the colour of a fat-soluble indicator. The most common of these are Nile Blue sulphate, Night Blue, Spirit Blue and Victoria Blue; but toxicity, variable dye composition or fading reactions (Lawrence, 1967*a*, *b*; Alford & Steinle, 1967) often cause problems. The need for flexibility in choosing substrates and for minimizing variations in surface area of fat available for lipase action led us to develop an overlay method for lipolysis (Alford & Steinle, 1967). Others (Fryer, Lawrence & Reiter, 1967; Muys & Willemse, 1965) have proposed similar methods. Chemical detection of the fatty acids liberated is usually by some extraction-titration procedure (Goldman & Rayman, 1952; Alford & Elliott, 1960) with identification by gas chromatography (Alford, Elliott, Hornstein & Crowe, 1961).

(c) *Factors affecting lipase activity*

In any attempt to determine lipase activity, several factors are important, whether measuring lipolysis in Petri dishes, determining fatty acids by titration or chromatography, or studying the role of lipase activity in foods.

(i) *Fat used as substrate*

Although lipases generally attack a wide variety of substrates, enough specificity has been shown to warrant consideration of the fat used (Alford & Steinle, 1967). It should at least reflect some of the triglycerides and fatty acids present in the product of interest. For example, using only tributyrin to study the lipolytic microflora of beef could cause erroneous conclusions.

(ii) *Temperature of incubation*

Temperatures above the optimum for lipase production inhibit its production before they affect culture growth (Nashif & Nelson, 1953; Alford & Elliott, 1960). The temperature of incubation of the enzyme-substrate mixture also is important and apparently affects specificity (Alford & Pierce, 1961). The shift toward increased unsaturation as temperature is decreased is illustrated in Table 1. This is probably related to the physical state of the substrate rather than a direct effect on the enzyme, but in either case it could play a role in the type of hydrolysis occurring in frozen foods or in products ripened at low temperatures. Caution is advisable, therefore, in extrapolating data obtained at 20–50° to what is happening in cold storage.

TABLE 1

Influence of temperature on type of fatty acids released by microbial lipases from coconut oil (after Alford & Pierce, 1961)

Lipase from	% of total free fatty acids* released as			
	Lauric acid at		Oleic acid at	
	35°	−7°	35°	−7°
Pseudomonas fragi	46	29	5	25
Geotrichum candidum	39	3	14	52
Penicillium roquefortii	46	35	4	24

* Conditions of incubation of enzyme–coconut oil mixtures and % of other free fatty acids released are given in the original paper.

(iii) *Composition of growth medium*

A high carbohydrate substrate will inhibit or reduce lipase production (Nashif & Nelson, 1953; Alford & Elliott, 1960) and the protein, peptides or amino acids used as sources of nitrogen are important considerations (Lawrence *et al.*, 1967; Alford & Pierce, 1963). Thus, it would appear likely that the carbohydrate and protein content of a food would affect lipase production in it and might be manipulated to control lipase activity.

(iv) *Oxygen availability*

Vigorous aeration decreases lipase production or at least its accumulation, yet growth in media with high surface/volume ratios or with slow agitation is stimulatory (Nashif & Nelson, 1953; Alford & Elliott, 1960; Alford & Smith, 1965; Lawrence *et al.*, 1967). Anaerobic lipase activity has been shown in rumen studies (Hobson & Summers, 1966). We have obtained evidence (unpublished) that oxygen-impermeable packaging may limit the degree of lipase activity in stored, ground meat.

(d) *Lipase specificity*

There is little or no evidence to suggest a specificity related to a specific fat (triglyceride). There is good evidence, however, of specificity related to the position of attachment of a fatty acid to the triglyceride molecule (Alford, Pierce & Suggs, 1964;

Mencher & Alford, 1967) and to the structure of the fatty acid being hydrolysed (Jensen *et al.*, 1965; Marks, Quinn, Sampugna & Jensen, 1968). Other lipases appear to have no position specificity. These data are illustrated in Table 2. The *Geotrichum*

TABLE 2

Determination of specificity of microbial lipases by their action on triglycerides of known composition (after Alford, Pierce & Suggs, 1964)

Lipase from	% composition of triglyceride			
	2-Oleoyl distearin		2-Stearolyl diolein	
	Oleic acid	Stearic acid	Oleic acid	Stearic acid
Geotrichum candidum	98*	2	99	1
Pseudomonas fragi	2	98	98	2
Staphylococcus aureus	25	75	63	37

* % of total free fatty acids released from enzyme–triglyceride mixture incubated at 35° for 1–3 h.

candidum lipase is specific for oleic acid (unsaturated fatty acids) regardless of position, the *Ps. fragi* lipase is specific for the 1-position regardless of the fatty acid, and the *Staph. aureus* lipase exhibits neither position nor fatty acid specificity. As pointed out by Lawrence (1967*a*, *b*), the number of carbon atoms in a fatty acid is of uncertain importance as an influence on hydrolysis by lipases because of emulsification difficulties. Jensen, Sampugna & Pereria (1964), in studies with pancreatic lipase and synthetic triglycerides, found no intramolecular specificity but did find intermolecular specificity. Their conclusions that some classes of milk triglycerides are more rapidly hydrolysed by pancreatic lipase than others would appear a reasonable assumption for some microbial lipases, since a close similarity has been demonstrated (Mencher & Alford, 1967). It is important to keep in mind, however, that the triglyceride structure as well as its component fatty acids is important (Table 3). By examining only the data from lard, one could say that *Pseudomonas fragi* and *Geotrichum candidum* were similar

TABLE 3

Influence of composition of fat on fatty acids released by different micro-organisms

Origin of fat	% of fatty acids in		
	Original fat	Hydrolysis by lipase of	
		Ps. fragi	*G. candidum*
Lard			
Palmitic acid	26	4	4
Oleic acid	27	68	73
Corn oil			
Palmitic acid	13	13	3
Oleic acid	28	27	42

in action. However, we know that in lard most of the palmitic acid is esterified at the 2-position. Because *Ps. fragi* lipase preferentially attacks the 1-position (Alford *et al.*, 1964) and *G. candidum* lipase preferentially attacks the *cis*-9 unsaturated bond (Marks *et al.*, 1968), the results are similar but for different reasons. In a fat such as corn oil, where the fatty acids are more randomly distributed, the differences are more apparent. Selective hydrolysis of fatty acids that are not randomly distributed in other fats, such as cacao, may offer possibilities for controlling flavour changes in foods.

(e) *Inhibition of lipase by fatty acids*

For microbial lipases to be of appreciable importance in foods, they must be produced and active in the presence of the substrate and the fatty acids released. Suggestions in the literature (Nashif & Nelson, 1953) and work in our laboratory (Smith & Alford, 1966) have shown that production and activity of some lipases are sensitive to accumulation of certain fatty acids. When 0·5% of lard (as an emulsion) is present during the growth of *Ps. fragi*, little lipolytic activity is detected. However, strong lipase activity is obtained in growth cultures when lard is omitted.

It is well known that fatty acids inhibit a variety of biological reactions and since the inhibition of lipase paralleled the appearance of FFA, the sodium salts of various fatty acids were prepared and tested to determine their inhibitory action on *Ps. fragi* lipase. Long chain, unsaturated fatty acids at a concentration of 1 μmole/ml were inhibitory whereas long chain, saturated fatty acids were not (Table 4). Those unsaturated fatty

TABLE 4

Effect of sodium salts of fatty acids on the activity of lipase produced by Pseudomonas fragi (*after Smith & Alford*, 1966)

Compound added	% inhibition of lipase after (h)	
	1	3
None	0	0
Sodium palmitoleate (16-1)*	88	93
Sodium oleate (18-1)	61	92
Sodium elaidate (18-1)	0	6
Sodium linolenate (18-3)	58	94
Sodium arachidonate (20-4)	64	91
Sodium palmitate (16-0)	0	14
Sodium stearate (18-0)	0	10
Sodium arachidate (20-0)	0	20

* No. of carbon atoms and unsaturated bonds in molecule.

acids that were inhibitory also decreased the surface tension from 51·7 to 30 dynes/cm or less. Subsequent studies, however, did not support this correlation. Because oleate is the major unsaturated fatty acid of lard, sodium oleate at 1 μmole/ml was selected for further study. Divalent metallic ions, such as Ca^{2+}, Sr^{2+}, Ba^{2+}, Cd^{2+}, Mn^{2+} and Co^{2+}, eliminated the oleate inhibition of lipase. A variety of chemically unrelated molecules, such as the Tweens, bovine serum, Triton X-100 and lecithins, also eliminated the inhibition of lipase by Na oleate.

Peck (1942) stated that Ca^{2+} eliminate oleate inhibition of trypsin action by formation of an insoluble soap. This type of action may explain the elimination of oleate inhibition to lipase by certain metallic ions, although Fe^{2+}, Zn^{2+} and Hg^{2+} also form insoluble soaps with fatty acids but were unable to eliminate oleate toxicity toward microbial lipase. The ineffective cations may have been more strongly complexed by other components of the medium.

The effect of lard and sodium oleate on other lipase systems is shown in Table 5. The effect of proteins on all lipases is also shown. It is well known that serum albumin combines with fatty acids (Davis & Dubos, 1947; Saifer & Goldman, 1961); thus, the ability of bovine serum to protect against oleate inhibition can be explained by a protein–fatty acid combination. It would appear, therefore, that in dairy foods as well as other foods with a reasonable level of calcium and protein, the inhibition of the lipase by unsaturated fatty acids would be minimized.

TABLE 5

Effect of lard and oleate on various lipolytic systems (after Smith & Alford, 1966)

Source of lipase	Inhibition of lipase activity by	
	Lard, 1%	Na oleate, 1 μmole/ml
Geotrichum candidum (protein medium)	29	0
Geotrichum candidum (synthetic medium)	39	92
Pseudomonas fragi (high protein medium)	76	42
Pseudomonas fragi (1% peptone medium)	85	91
Staphylococcus aureus (protein medium)	31	0
Pancreatic lipase	8	0

3. Oxidative Changes

(a) *Definition*

Rancidity is the oxidative deterioration of food lipids, and involves the reaction of the unsaturated fatty acids with oxygen to give hydroperoxides which in turn decompose to products with undesirable taste and odour. Many of these undesirable compounds are aldehydes. Micro-organisms decompose cumene hydroperoxide, *tert*-butyl hydroperoxide, *p*-menthane hydroperoxide (Updegraff & Bovey, 1958), and 1-alkyl-hydroperoxides (Finnerty, Kallio, Klimstra & Wawzonek, 1962) as well as aldehydes (Racker, 1950; Black, 1951; Seegmiller, 1953; DeMoss, 1954). However, our reports (Smith & Alford, 1968, 1969) are the only ones of which we are aware in which the action of a large number of micro-organisms on rancid and fresh fat have been investigated. Cantoni and his co-workers have studied the changes in the microbial flora and lipids during dry sausage ripening (Cantoni, Molnar, Renon & Giolitti, 1966, 1967*a*, *b*). They isolated and identified a large number of fatty acids and carbonyls that may be involved in sausage flavour.

Microbial lipolysis

(b) *Microbial action on rancid fat*

(i) *Peroxides*

In Fig. 1 the effect of micro-organisms on the peroxides of rancid lard is shown. The effect varied from 18% decomposition by *Ps. ovalis* and *Streptomyces* spp. to 100%

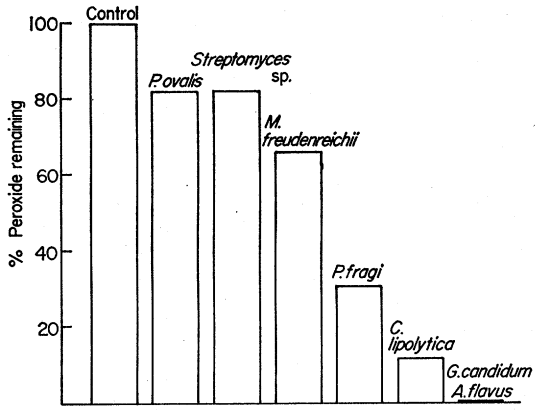

Fig. 1. The effect of micro-organisms on the peroxides of rancid lard. The initial peroxide values ranged 74·3–97·4 meq/kg of fat (mean, 82·9 meq).

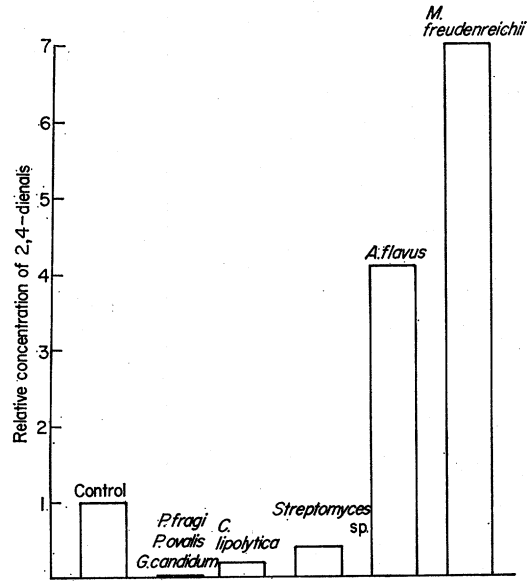

Fig. 2. The effect of micro-organisms on the 2,4-dienals of rancid lard. The 2,4-dienal concentration of the controls ranged 1·5–3·4 μmoles/10^4 μmoles fat (mean, 2·0 μmoles).

decomposition of the peroxides by *G. candidum* and *Aspergillus flavus*. Fifteen of the 29 cultures studied decreased the peroxide value by 50% or more in the 5 days' incubation period.

(ii) *Monocarbonyl compounds*

The effect of the micro-organisms on the monocarbonyl compounds (2,4-dienals, 2-enals, and alkanals) was quite varied and complex. *Pseudomonas fragi*, *Ps. ovalis*, and *G. candidum* destroyed the 2,4-dienal fraction completely (Fig. 2), while *Asp. flavus* and *Micrococcus freudenreichii* increased the dienal content 4- to 7-fold. Fifteen of the 29 cultures decomposed the dienals while 5 cultures increased the dienal concentration by 2-fold or more.

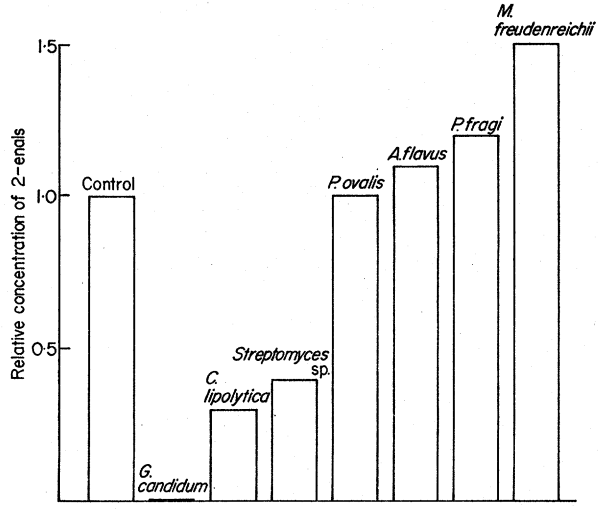

Fig. 3. The effect of micro-organisms on the 2-enals of rancid fat. The 2-enal concentration of the controls ranged 3·6–6·7 μmoles/10^4 μmoles fat (mean, 5·0 μmoles).

In Fig. 3, the effect of micro-organisms on the 2-enal portion of the monocarbonyls is shown; *G. candidum* destroyed this class completely (this organism also decomposed the 2,4-dienal class) but no culture produced a great increase in enal concentration comparable to that obtained with the dienals. Ten of the 29 cultures destroyed the enal class (these same organisms also decomposed the dienals); 2 cultures of the 29 increased the 2-enal concentration by a factor of 2.

The effect of micro-organisms on the alkanal class varied from a sharp decrease with *Streptomyces* spp. to a large increase with *G. candidum*. Six of the 29 cultures increased the alkanal content by 2-fold or more; 4 cultures decreased this class by at least 50%.

There was no relationship between the disappearance of peroxides and the changes in monocarbonyls. The products of peroxide decomposition are unknown and vary with the micro-organism. *Aspergillus flavus*, for example, destroyed peroxides and produced large increases in monocarbonyls, whereas *C. lipolytica* decomposed peroxides but showed a decrease in the monocarbonyl content. The experiments of Lilly, Smith

& Alford (1970) indicate that the peroxide and monocarbonyl destruction by micro-organisms is an enzymic process. There was no activity when the microbial fraction was boiled and the activity was found to be dependent on pH, temperature and enzyme-substrate ratio. The data also suggest that the enzymes that decompose peroxides are not the same as those that decompose the monocarbonyl compounds.

The ability of micro-organisms to attack selectively specific compounds in rancid lard suggests that a wide range of flavour effects could be obtained by selection of cultures which produce or remove volatile carbonyls in fat-containing foods. It may even be possible to remove peroxides and carbonyls from a rancid fat and thus reclaim that fat for use.

(c) *Effect of oxidized fat on ground pork*

Because certain oxidized fats have been shown to affect spore germination (Roth & Halvorson, 1951), the effect of rancid fat on the spoilage of ground pork was examined. Fat was added to 2 lots of freshly cubed pork to give a concentration of 30%. To one lot was added fresh fat and to the other sufficient rancid (by UV light) fat to give an initial peroxide value of *c.* 15. These lots were ground, packaged, and stored at 5°. At intervals the plate count, FFA, peroxide value, proteolysis as measured by ninhydrin (Saffle, May, Hamid & Irby, 1961), resazurin reduction (Saffle *et al.*, 1961) and odour were determined. In one experiment there was a delay in off odour development in the presence of the slightly rancid lard, but this could not be repeated in subsequent experiments. Proteolysis, microbial growth and further changes in FFA were not significantly different with the 2 treatments.

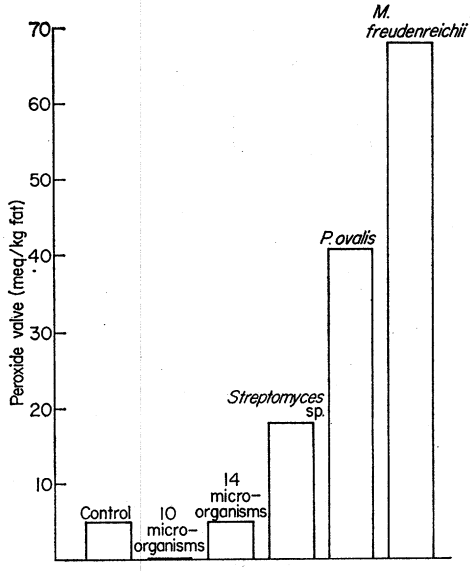

Fig. 4. The effect of micro-organisms on peroxides in fresh lard.

(d) *Microbial action on fresh fat*

(i) *Peroxides*

Ten of 28 micro-organisms destroyed the small amount of peroxide present in fresh lard while 14 had no effect (Fig. 4). Two strains of *Streptomyces* increased the peroxide concentration *c.* 3-fold, *Ps. ovalis* increased the concentration by 8-fold, and *M. freudenreichii* increased the peroxides *c.* 14-fold.

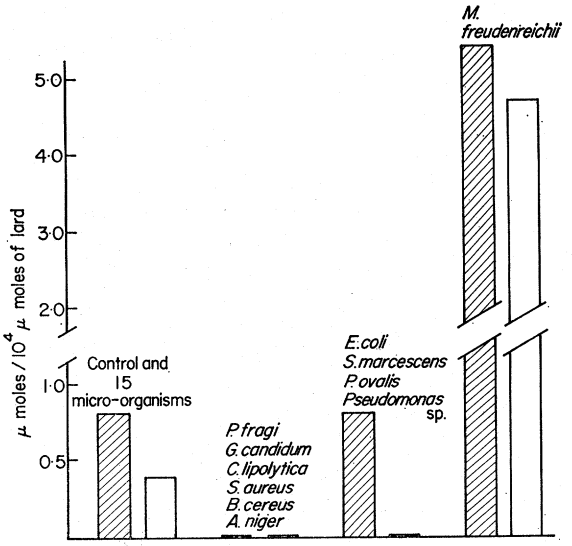

Fig. 5. The effect of micro-organisms on 2-enals and 2,4-dienals in fresh lard. Hatched columns, 2-enals; open columns, 2,4-dienals.

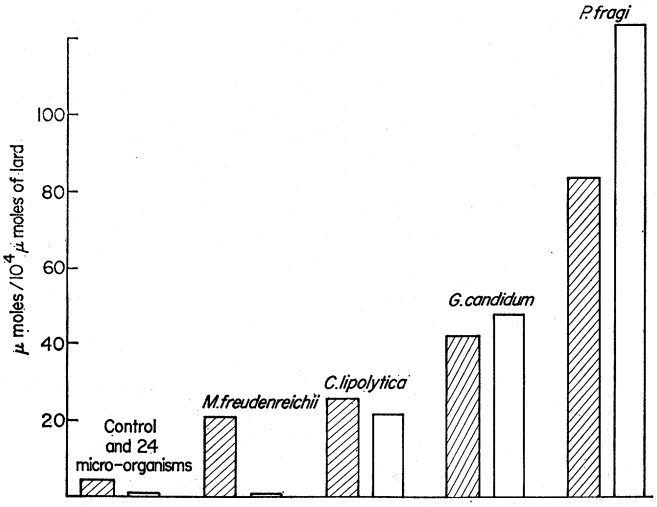

Fig. 6. The effect of micro-organisms on alkanals and methyl ketones in fresh lard. Hatched columns, alkanals; open columns, methyl ketones.

(ii) *Monocarbonyl compounds*

Micrococcus freudenreichii produced a large increase in the concentration of 2,4-dienals and 2-enals (Fig. 5). Most of the micro-organisms utilized in the study had no effect on the unsaturated classes of the monocarbonyls; however, 8 cultures completely decomposed these classes, while 4 removed only the 2,4-dienals.

Most of the micro-organisms had little effect on the alkanal fraction (Fig. 6); *Ps. fragi*, *G. candidum*, and *C. lipolytica* increased the concentration of the alkanals as well as producing methyl ketones, a fraction that is not present in fresh lard. *Micrococcus freudenreichii* showed an increase in alkanals but did not produce methyl ketones.

The acceleration of peroxide formation and the increase in the 3 classes of monocarbonyl fraction by *M. freudenreichii* suggest that micro-organisms can carry out reactions involved in the production of rancidity, i.e. the production of peroxides with their subsequent decomposition to monocarbonyls, as suggested by Lea (1938) and Jensen (1954). However, this ability to produce peroxides and monocarbonyls appears to be relatively rare among micro-organisms. The *Streptomyces* spp. and *Ps. ovalis*, which produced an increase in peroxides without a concomitant increase in the carbonyl fraction, either (a) lack the mechanisms necessary to convert peroxides to monocarbonyls, (b) decompose the monocarbonyls as rapidly as they are formed or (c) convert the peroxides to other compounds that were not determined.

The ability of *Streptomyces* spp., *Ps. ovalis*, and *M. freudenreichii* to form peroxides suggests that lipoxidase-like activity is present. It has been reported that micro-organisms produce lipoxidase (Mukherjee, 1951; Fukuba, 1952; Shimahara, 1966), but Tappel (1963) stated that there is no evidence for a microbial lipoxidase.

The precursors for methyl ketone production by *Ps. fragi*, *G. candidum*, and *C. lipolytica* are unknown, but these organisms are strongly lipolytic and fungi are known to produce methyl ketones by β-oxidation and decarboxylation of lipase liberated fatty acids (Hawke, 1966).

Castell & Garrard (1941) and Jensen (1954) suggested that micro-organisms that are both active in producing lipase and have a positive oxidase reaction are responsible for rancidity in fats. However, in this study, there was no correlation between ability to

TABLE 6

Relationships of oxidase reaction, lipase production, and oxidative activity by micro-organisms on lard (after Smith & Alford, 1969)

Effect on fresh lard	No. of cultures giving					
	Lipase production (μeq FFA/h) %				Oxidase reaction	
	>50	10–50	1–10	0	+	–
Increase peroxides, 2-enals, 2,4-dienals, and alkanals	0	0	1	0	0	1
Increase peroxides only	0	0	3	0	1	2
Destroy peroxides, 2-enals, 2,4-dienals; alkanals unchanged	2	2	0	1	0	5
Destroy 2,4-dienals only	0	0	4	0	2	2
None	3	0	6	4	0	13

produce lipase and/or a positive oxidase reaction and the degree of fat oxidation (Table 6).

4. Discussion

Most of the evidence suggesting possible roles of micro-organisms in lipolytic changes in foods is derived from data using model systems rather than natural foods. Nevertheless, it would appear that the production and destruction of peroxides and monocarbonyl compounds could be manipulated by selection of micro-organisms to bring about the changes desired. Considerable variation in microbially induced changes in naturally contaminated foods can be expected since different species among the normal contaminants might vary widely in the types of changes they produce. Fat hydrolysis as determined by lipase production can be manipulated by altering the environment to influence the amount produced. Except in instances of products containing volatile fatty acids, however, it is probable that lipase activity by itself is important in flavour changes only as a first step in a series of reactions. There is no correlation between lipase production and the oxidative activities of micro-organisms. Thus, whether counts of lipase producers are a good measure of the total microbial potential for lipolytic changes is debatable.

As techniques for isolation and identification of the products of oxidation become more reliable and easier to perform on a routine basis, carefully controlled experiments involving foods containing known micro-organisms should provide more definitive data on the specific roles of micro-organisms in the changes in fats.

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